JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2003, p. 826–830 Vol. 41, No. 2
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.2.826–830.2003

Identification of Dermatophyte Species by 28S Ribosomal DNA Sequencing with a Commercial Kit

Béatrice Ninet,[1] Isabelle Jan,[1] Olympia Bontems,[2] Barbara Léchenne,[2] Olivier Jousson,[2] Renato Panizzon,[2] Daniel Lew,[1] and Michel Monod[2]*

Division des Maladies Infectieuses, Hôpital Cantonal Universitaire, 1211 Geneva,[1] and Service de Dermatologie (DHURDV), Centre Hospitalier Universitaire Vaudois, 1012 Lausanne,[2] Switzerland

Received 6 May 2002/Returned for modification 23 September 2002/Accepted 26 November 2002

We have shown that dermatophyte species can be easily identified on the basis of a DNA sequence encoding a part of the large-subunit (LSU) rRNA (28S rRNA) by using the MicroSeq D2 LSU rRNA Fungal Sequencing Kit. Two taxa causing distinct dermatophytoses were clearly distinguished among isolates of the *Trichophyton mentagrophytes* species complex.

Dermatophytes are the main cause of superficial mycoses (9, 15, 16). These fungi have the capacity to invade keratinized tissue of humans or animals to produce infections that are generally restricted to the corneocytes of the skin, hair, and nails. Among the approximately 10 species isolated in Europe, *Trichophyton rubrum* and *T. mentagrophytes* are the most commonly observed, with frequencies varying from 27 to 74% and from 17 to 41%, respectively (13).

Dermatophytes are usually identified on the basis of macroscopic appearance, together with microscopic examination of cultures. Important characteristics are the rate of growth, the shape and texture of the culture on solid media, color, diffusion of pigments into agar, and sporulation. However, identification of dermatophytes often remains difficult or uncertain because there are variations from one isolate to another. Recent advances in molecular biology and progress in technology have allowed the development of new techniques for species determination and strain typing in microbiology. The molecular approach used to identify fungi is often based on sequence analysis of the ribosomal DNA (rDNA) and in particular on the internal transcribed spacer (ITS). The polymorphism of the ITS1 and ITS2 regions flanking the DNA sequence encoding the 5.8S rRNA was previously shown to be suitable for the identification of clinically important yeasts (1), *Aspergillus* sp. (8),and dermatophyte species (3–5). In contrast, the gene coding for the small-subunit rRNA (18S rRNA) did not discriminate sufficiently between dermatophyte species (7).

The MicroSeq D2 large-subunit (LSU) rRNA Fungal Sequencing Kit (Applied Biosystems, Rotkreuz, Switzerland) was recently developed to identify fungal species after amplification of a partial sequence of the DNA encoding the LSU rRNA (28S rDNA). The sequence of a given fungus can then be compared for identification with the rDNA sequences of the MicroSeq D2 Fungal database, which includes more than 500 validated sequences from different fungal species but not from dermatophytes. In the present study, we tested the MicroSeq D2 LSU rRNA Fungal Sequencing Kit by using it to identify dermatophyte species from patients referred to the mycological laboratory of the Department of Dermatology at the University Hospital in Lausanne, Switzerland (Table 1). Neotypes of different species and other reference strains (Table 2) were used for comparison of DNA sequences. This study allows the extension of the MicroSeq D2 Fungal database to the determination of dermatophytes.

Isolates. Skin and nail scrapings and hair fragments were collected from patients with suspected mycoses. Routinely, one part of each sample was examined in Na_2S dissolvent with Blankophor under a fluorescence microscope to detect fungal elements (11, 12) and in parallel, another part was seeded in test tubes containing Sabouraud's agar medium with chloramphenicol and with chloramphenicol plus cycloheximide. The cultures were incubated at 30°C. Dermatophytes were identified after 14 to 21 days following macroscopic and microscopic examinations (9, 10, 15). When necessary, the determination of *Microsporum canis* was confirmed after inoculation on lactritmel agar, where the fungus produces a large amount of characteristic, spindle-shaped macroconidia (10). Isolates were documented as *T. mentagrophytes* on the basis of the production of clusters of pyriform microconidia.

DNA extraction. DNA was extracted from fresh dermatophyte cultures on Sabouraud's agar medium. Approximately 1 cm^2 of mycelium was collected and introduced into an Eppendorf tube containing 1 ml of distilled water and 12 to 15 glass beads with a diameter of 3 mm (Merck). After vortexing at maximum speed for 2 min, 100 μl of a suspension containing fragmented mycelium was transferred to a second tube containing an equal volume of <106-μm glass beads (Sigma). The mycelium was further disrupted for 5 min by shaking in a disintegrator (Mickle Laboratories, Gomshall, United Kingdom), followed by three subsequent steps of freezing in liquid nitrogen and heating at 95°C for 5 min. After centrifugation, 35 μl of supernatant was mixed with 150 μl of 100% ethanol and loaded onto a QIAamp DNA mini kit column (Qiagen, Basel, Switzerland). The DNA was purified by following the protocol provided by the manufacturer and eluted with 200 μl of distilled water. One microliter of the DNA suspension was used for PCR amplification.

* Corresponding author. Mailing address: Service de Dermatologie, Laboratoire de Mycologie, BT422, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland. Phone: 41 21 314 0376. Fax: 41 21 314 0378. E-mail: Michel.Monod@chuv.hospvd.ch.

TABLE 1. Localization of dermatophyte isolates analyzed in this study

Source	No. of isolates of:										
	T. mentagrophytes			*T. tonsurans*	*T. rubrum*	*T. soudanense*	*T. violaceum*	*M. canis*	*M. audouinii*	*M. gypseum*	*E. floccosum*
	Type I	Type II	Type III								
Human											
Tinea pedis	15	5			2						
Tinea unguium	11	11			16		1				1
Tinea manuum			3		3						
Tinea corporis		1	8		2	2	1	2		4	
Tinea cruris					2			1			1
Tinea facie			5	1				1			
Tinea barbae			1								
Tinea capitis			5	9		6	2	4	5		
Dog			1								
Cat			1								
Total	26	17	24	10	25	8	4	8	5	4	2

PCR and DNA sequencing. Amplification of 28S rDNA was performed by using the primers and the PCR mixture included in the MicroSeq D2 LSU rDNA Fungal Sequencing Kit. This kit provides all of the reagents necessary to amplify and also to sequence the D2 expansion DNA segment region encoding the nuclear LSU rRNA. Thirty microliters of each PCR product was purified with the QIAquick purification PCR kit (Qiagen) and eluted in 30 μl of the E buffer provided in the purification kit. Five microliters was used for direct sequencing with an automated ABI Prism 377 DNA sequencer (Applied Biosystems) in accordance with the protocol supplied by the manufacturer. rDNA sequences were aligned with the Sequence Navigator program (version 1.0) and analyzed by using BioEdit version 5.0.9 (6).

Amplification of the ribosomal ITS region was performed as previously described (4), by using primers 5′-GGTTGGTTTC TTTTCCT-3′ and 5′-AAGTAAAAGTCGTAACAAGG-3′.

PCR identification of dermatophytes. PCR amplifications of the 28S rDNA fragment of dermatophyte species gave a single product of 312 to 314 bp (Fig. 1). The selected sequence was unique and species specific for all isolates of *T. rubrum*, *T. tonsurans*, *T. soudanense*, *T. violaceum*, *M. canis*, *M. audouinii*, *M. gypseum*, and *Epidermophyton floccosum*. Reference strains designated neotypes of *T. tonsurans*, *M. canis*, and *M. audouinii*, as well as reference strains of *T. rubrum*, *T. violaceum*, and *E. floccosum*, showed sequences identical to those of our isolates. Three different sequences designated types I, II, and III for further investigations were detected for 26, 17, and 24 *T. mentagrophytes* isolates, respectively. Reference strain CBS 428.63, designated the neotype of *T. interdigitale* (4), was type I. *T. mentagrophytes* type IV was reserved for the sequence of strain CBS 318.56, which was designated the neotype of *T. mentagrophytes* (4).

The differences between the species were generally due to single-nucleotide polymorphisms. The interspecific sequence divergences ranged from 0.4% (between *T. tonsurans* and *T. mentagrophytes*) to 4.5% (between *T. tonsurans* and *M. audouinii*). *T. mentagrophytes* types I, II, and III were distinguished on the basis of two polymorphic sites located at positions 99 and 139 of the partial 28S rDNA sequence alignment (Fig. 1).

The occurrence of a species complex within *T. mentagrophytes* has been previously suggested by PCR fingerprinting (2), amplified fragment length polymorphism analysis, and rDNA sequencing (3–5). We searched by ITS sequencing to determine whether or not the observed polymorphism in the 28S rDNA was representative of intraspecific taxa. Two different ITS sequences (AF506033 and AF506036) were found in type I and II *T. mentagrophytes* isolates. The AF506033 and AF506036 sequences differ by a single deletion at position 418 (Fig. 2). Strain CBS 428.63, designated the neotype of *T. interdigitale*, showed the AF506033 sequence. A unique ITS sequence (AF506034) was found in type III *T. mentagrophytes*

TABLE 2. Reference strains used in this study

Strain	Source	Comment	Reference(s)
T. rubrum CBS 392.58	Unknown	Used as reference strain of *T. rubrum*	3, 4
Arthroderma vanbreuseghemi CBS 428.63[a]	Tinea pedis	Designated neotype of *T. interdigitale*	4
T. mentagrophytes CBS 318.56	Suppurative infection	Designated neotype of *T. mentagrophytes*	4
T. tonsurans CBS 496.48	Tinea capitis	Designated neotype of *T. tonsurans*	4
T. violaceum CBS 459.61	Unknown		
Arthroderma otae CBS 496.86[b]	Cat	Designated neotype of *M. canis*	5
M. audouinii CBS 545.93	Tinea capitis	Designated neotype of *M. audouinii*	5
E. floccosum CBS 970.75	Onychomycosis	Used as reference strain of *E. floccosum*	5

[a] Teleomorph of *T. interdigitale*.
[b] Teleomorph of *M. canis*.

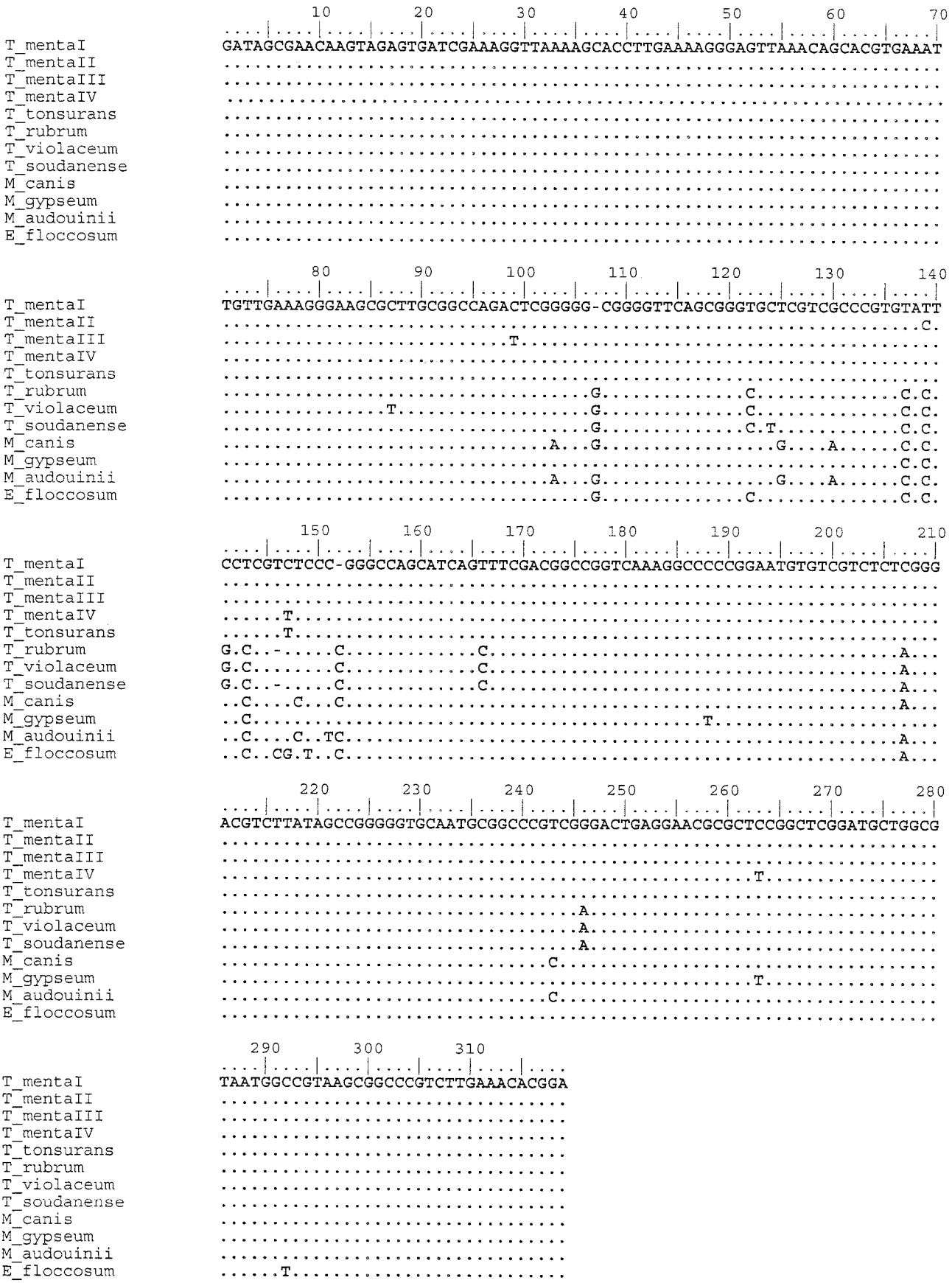

FIG. 1. Multiple alignment of partial 28S rDNA sequences of *Trichophyton* (T), *Microsporum* (M), and *Epidermophyton* (E) species. A dash indicates an alignment gap; a dot indicates the same base as on the upper line.

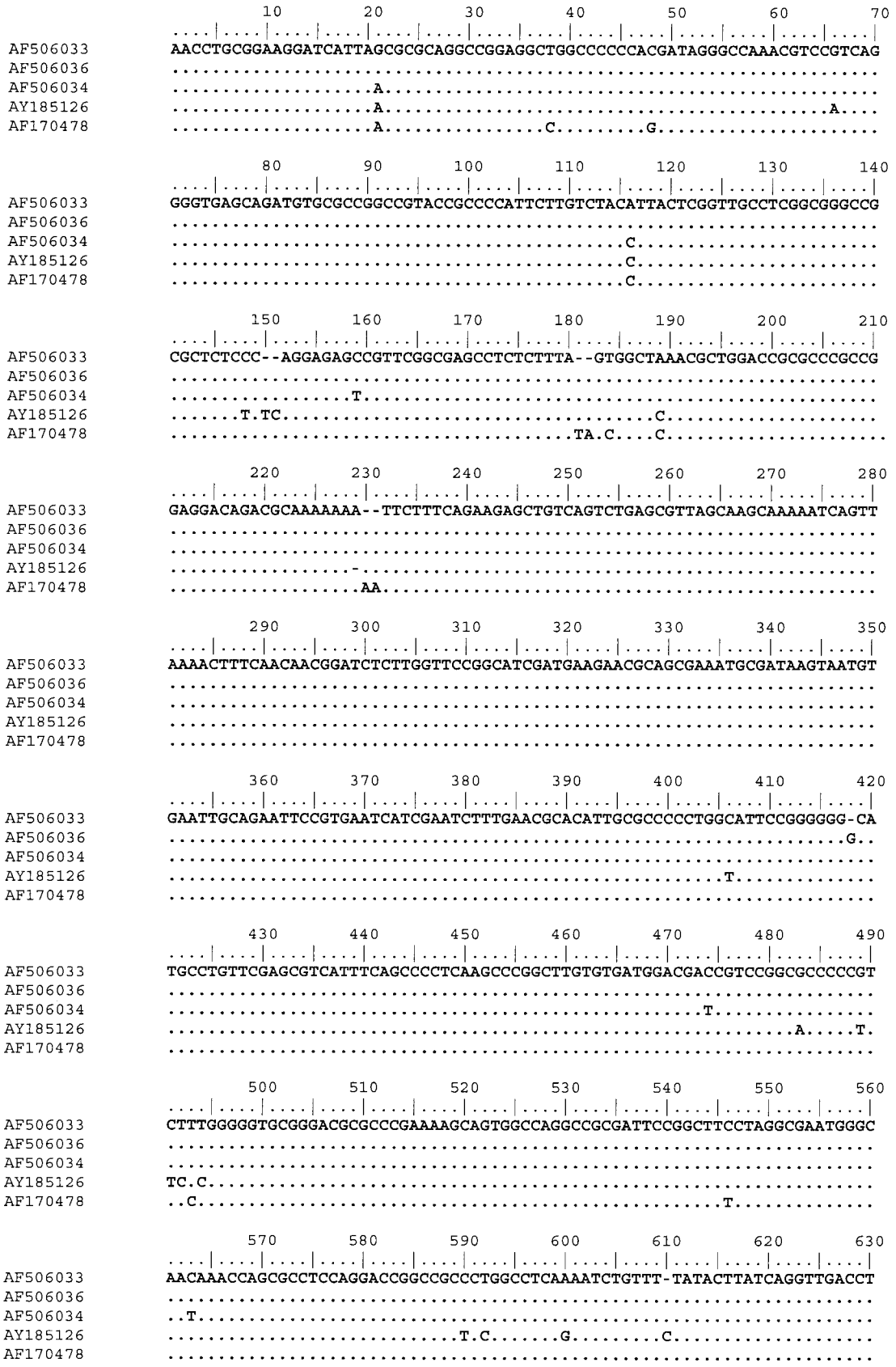

FIG. 2. Alignment of multiple complete ITS rDNA sequences (ITS1 plus 5.8S plus ITS2) of *T. mentagrophytes* types I, II, III, and IV (accession no. AF506033, AF506036, AF506034, and A4185126, respectively) and *T. tonsurans* (AF170478). The sequence with accession no. AF506033 was found in four isolates of *T. mentagrophytes* type I and one isolate of type II. The sequence with accession no. AF506036 was found in three isolates of type I and two isolates of type II. The sequence with accession no. AF506034 was found in the five isolates of *T. mentagrophytes* type III tested. ITS1, bp 23 to 284; 5.8S, bp 285 to 440; ITS2, bp 441 to 623.

isolates. This ITS sequence differs from the ITS sequences of type I and II isolates by five nucleotide substitutions (four transitions and one transversion), two of which are shared with *T. tonsurans*. This indicates that *T. mentagrophytes* type III is divergent from types I and II. None of the dermatophyte ITS sequences previously published (14) corresponded to that of *T. mentagrophytes* type III. The neotype of *T. mentagrophytes* showed a more divergent sequence (accession no. A4185126) that differs by 14 or 15 nucleotide substitutions and four insertions/deletions from the sequences with accession no. AF506033, AF506036, and AF506034 (Fig. 2).

The amount of microconidia was variable in isolates of types I and II. Isolates of type III were characterized by a faster-growing mycelium with a powdery appearance due to abundant quantities of microconidia. The presence of filaments in spirals was observed in some isolates but not in all three types of *T. mentagrophytes*. The neotype *T. mentagrophytes* (type IV) culture was very similar in appearance to those of type I and II isolates.

It is known that each dermatophyte species has a predilection for certain body areas. For instance, *T. rubrum* is especially dominant in onychomycoses whereas *M. canis* is especially prevalent in tinea capitis and tinea corporis (13). In contrast, some species of dermatophytes are never or rarely isolated from a particular dermatophytosis. Retrospective investigations revealed that type I and II *T. mentagrophytes* isolates were from tinea pedis, like the neotype of *T. interdigitale*, or tinea unguium, whereas 22 type III *T. mentagrophytes* isolates were from other tineas (Table 1). Two other type III isolates were from a cat and a dog.

In conclusion, two taxa were distinguished among the *T. mentagrophytes* strains we have isolated (Table 1). In the first taxon belong the type I and II strains corresponding to the fungus described as *T. interdigitale* (4), *T. mentagrophytes* var. *interdigitale* (9), or *Microides interdigitale* (15). This taxon was reported only from humans. The second taxon, to which the type III strains belong, corresponds to the fungus described as *T. mentagrophytes* var. *mentagrophytes* (9) or *Microides mentagrophytes* (15) and reported from animals and humans. These two taxa cause distinct dermatophytoses in humans (Table 1). Reference strain CBS 318.56, designated the neotype of *T. mentagrophytes* (4), is likely to belong to another taxon.

The 28S rDNA is the target of choice in the development of a method for rapid identification of dermatophytes with high specificity and sensitivity. At present, in clinical laboratories, a fungal species causing an infection can only be identified after growing in culture for 2 to 3 weeks. Rapid identification by PCR is particularly helpful in cases of tinea capitis, where the knowledge of the exact species of dermatophyte in clinical samples is needed before prescribing the appropriate treatment (10). The sequence of an unknown fungus isolated from dermatological samples can now be routinely compared with rDNA sequences from the EMBL GenBank database. The MicroSeq D2 Fungal database contains essentially rDNA sequences from environmental and plant pathogenic fungi and does not allow the identification of dermatophytes. These two databases could be further extended and completed with the genes of other dermatophyte species less important in human mycology and of species encountered in veterinary medicine.

Nucleotide sequence accession numbers. The dermatophyte 28S rDNA sequences described in this study have been deposited in the GenBank database and assigned accession no. AF378734 (*T. rubrum*), AF378735 (*T. soudanense*), AF378736 (*E. floccosum*), AF378738, AF378739, AF378740, A4185127 (*T. mentagrophytes* types I, II, III, and IV, respectively), AF448547 (*T. tonsurans*), AF448549 (*M. langeronii*), AF448550 (*M. canis*), AF448551 (*M. gypseum*), and AF506035 (*T. violaceum*).

We thank Mary Holdom and Harold Pooley for critical review of the manuscript and assistance with the English.

This work was partially supported by the Swiss National Foundation for Scientific Research (grant 3200-63687).

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